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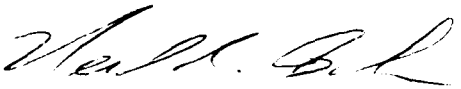
Investigator: Neil R. Baker, Assistant Professor, Department of Microbiology, The Ohio State University

Mailing address: 484 W. 12th Ave., Columbus, OH 43210

Signature of Principle Investigator:



Final Report

The purpose of this project has been to produce non-toxic immunogenic fragments of exotoxin A from Pseudomonas aeruginosa for use in a vaccine. Initially, three phases of the project were identified. Phase I involved screening proteolytic agents for their ability to fragment the toxin and identifying non-toxic fragments. Phase II involved scaling-up the operation to produce larger quantities of non-toxic fragments and immunization of animals. Phase III deals with evaluation of immunization against the non-toxic fragments of the toxin using a burned mouse model. To date we have been successful in fragmenting the toxin with several agents under a variety of conditions. However, we have not been able to show that any of the fragments are immunogenic, although they clearly are not toxic. The lack of immunogenicity may be due to the treatments used in generating the fragments. Thus we have been concentrating our efforts on finding new methods of fragmenting and purifying potentially useful fragments.

Summary of fragmentation data - Early in the project we found that most proteolytic agents did not alter the toxin molecule unless the toxin had been modified. Some proteases, such as proteinase K, acted on the native toxin, but completely digested the toxin and thus were not useful. Treatment with trypsin, papain and thermolysin produced the most useful results. Trypsin alone at 8 ug/ml cleaved a small fragment from the toxin but did not alter the activity of the toxin. If the toxin was treated with 0.1% SDS followed by treatment with trypsin at 2 to 8 ug several fragments with M.W. of 41K, 39K, 37.5K, 36K and 33K were observed by SDS-PAGE. The band at 39K was predominant in these preparations. Prior treatment with DTT or DTT

and SDS did not produce additional bands. The 41K and 39K fragments appear to have retained ADPR-transferase activity but the others are low in activity or inactive. Analysis of the SDS-trypsin treated preparation reveals a loss of ADPR-transferase activity indicating that much of the toxin has probably been destroyed. Similar results were produced by treatment of the toxin with SDS and an immobilized trypsin preparation.

Toxicity tests revealed that the toxin was inactivated following the SDS-trypsin treatment. Injection of mice intraperitoneally with 150 LD_{50} units of toxin or its equivalent resulted in no deaths in animals injected with SDS-trypsin treated toxin, whereas SDS treated toxin, trypsin treated toxin and untreated toxin were rapidly fatal.

Ouchterlony immunodiffusion analysis of SDS-trypsin treated toxin did not reveal any bands of precipitation although SDS treated and trypsin treated toxin produced clear bands of precipitation with specific antiserum.

Several attempts were made at immunizing mice and boosting anti-toxin titers in pre-immunized rabbits with SDS-trypsin treated toxin. All attempts were unsuccessful. The failure to show antigenicity by immunodiffusion and immunogenicity by immunizing animals indicated that we needed a new approach to the problem.

A possible explanation for our results was that SDS treatment was too harsh and has altered the immunogenicity of the toxin. Treatment with a mild, non-ionic detergent, Tween 20, followed by trypsin produced a pattern of fragmentation similar to that seen with SDS-trypsin. These fragments have not been analyzed for antigenicity or immunogenicity.

Papain and thermolysin also produce fragments of toxin under

appropriate conditions. Papain produces fragments with M.W. of 41K and 37K if the sample is treated with SDS and DTT. It is likely that the DTT is actually activating the papain rather than exposing additional cleavage sites on the toxin. Analysis of these fragments has not been done because the yield of fragments is not high. Thermolysin at 0.5 ug/ml will cleave the toxin into 6 fragments with M.W. of 61K, 56K, 52K, 50K, 40K and 36K. However, the yield is not good and these have not been analyzed further.

Recent work and progress

Currently a new approach has been taken, based upon the hydrophobic nature of the toxin. Exotoxin A clearly has two distinct functional areas, the A fragment which possesses enzymatic activity but is non-toxic alone, and the presumed B fragment which is involved in binding to the cell membrane. The A fragment has been purified and studied but the B fragment of exotoxin A has not been purified. In diphtheria toxin the B fragment is hydrophobic, a characteristic which has led to purification of the toxin by hydrophobic chromatography. Clearly, antibodies directed against the B fragment should protect against the lethal action of the toxin. The B fragment alone would be non-toxic since it does not have ADPR-transferase activity. Therefore, current investigations are studying the interactions of toxin A with hydrophobic gels. The initial experiments have been attempting to determine if the toxin binds to hydrophobic matrices and the conditions of elution of the toxin from the matrix. Presumably, the B segment of toxin bound to the hydrophobic gel would be protected from proteases and the binding may result in the exposure of cleavage sites for proteases. Thus, bound toxin could be treated with trypsin, washed and the B fragment could be eluted.

In order to determine if hydrophobic chromatography could be used to purify the toxin and possibly the B fragment, 200 ug of toxin was applied to 1 ml agarose columns with carbon side chains 0, 2, 4, 6, 8 and 10 carbons in length. All of the columns with carbon side chains bound toxin; the control column did not bind the toxin. Toxin could be eluted from the 2, 4 and 6 carbon columns with 0.25 M NaCl, the 8 carbon column with 0.5 M NaCl but could not be recovered from the 10 carbon column. Further analysis has shown that the toxin binds best to the C-6 column and that it can be eluted with 0.1 M NaCl. The purified toxin has little ADPR-transferase activity unless it has been treated with SDS and DTT which activates the toxin. The C-8 column binds the toxin more tightly. Elution requires 0.2-0.3 M NaCl and the toxin appears normal in that it also must be activated in order to show ADPR-transferase activity. The toxin binds very well to the C-10 column and cannot be eluted with NaCl. Other agents have not been tested for elution of the toxin.

Presumably the B segment of the toxin is involved in binding to the hydrophobic columns and it should be protected against the action of proteolytic enzymes. Currently experiments are in progress which will determine if the immobilized toxin can be cleaved by proteolytic enzymes to release the A fragment. The remaining fragment could be eluted and purified.

Currently, the rationale for continuing these experiments as a means of developing a vaccine is not valid. Iglewski et. al have developed a mutant strain which produces a non-toxic immunogenic form of the toxin. Thus, the non-toxic protein could be used in a vaccine and should be much easier to produce. However, the studies will be continued in order to produce fragments for sequencing the toxin and

in order to study the B fragment. Studies on the B fragment may provide insight into the binding and entry of toxin into the cells. It will also be possible to compare the P. aeruginosa fragment B to the diphtheria toxin fragment B and determine if they may have had a common evolutionary origin.

In summary, using several procedures we have been able to fragment exotoxin A into several peptides. The fragments are non-toxic but do not induce the formation of antibodies that react with native exotoxin A. The fragments also do not react with antibody to native toxin. The toxin can also be purified by hydrophobic chromatography. This property may provide a means of selectively cleaving the A fragment from the toxin. It may even be possible to use general proteases such as protease K to digest all portions the molecule which are not bound to the hydrophobic region of the support. If the toxin binds to the column by the same region that binds to the cell membrane then only the binding site will be left on the column. This can be eluted, purified and studied in more detail.